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# Subversion of Immune System by Tumor Cells and Role of Prostaglandins

**(immune surveillance/immunotherapy of cancer/prostaglandin synthetases/indomethacin)**

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**ABSTRACT** **Mice bearing syngeneic tumors, chemical and virus-induced, became immunologically unresponsive to sheep erythrocytes. The increase in the degree of unresponsiveness with tumor growth suggested a causal relationship. Immunosuppression was in fact caused by the tumor cells because the addition of tumor cells to *in vitro* cultures of spleen cells and sheep erythrocytes resulted in suppression of antibody response. Suppression was dose dependent with a ratio of 1 to 1000 of tumor cells to spleen cells sufficient to produce significant suppression. Prostaglandins were found to have a role in immunosuppression by tumor cells in that $PGE_2$ was itself immunosuppressive and in that indomethacin and aspirin, inhibitors of prostaglandin synthetases, blocked immunosuppression *in vitro* and retarded tumor growth *in vivo*. These findings suggest that tumors, although antigenic, may be able to escape immuno-surveillance by their host by means of subverting the immune system. Thus, success of immunotherapy may well depend on our ability to prevent or block the immunosuppressive activity of tumors.**

Tumor immunologists were first concerned with the question of whether or not tumors possess tumor-specific antigens. Now they ask why autochthonous and syngeneic tumors, possessing tumor-specific transplantation antigens, are not rejected by their hosts as homografts, and they seek to uncover mechanisms by which tumors escape destruction by the immune system.

Over the years much evidence has accumulated linking immunodeficiency and cancer, a subject recently reviewed by Kersey *et al.* (1). This association suggests the possibility that tumors may escape from host immune surveillance by subverting the immune system directly. We now have direct evidence, presented here, that syngeneic mouse tumors, both chemical and virus-induced, are indeed immunosuppressive. Additional evidence was published recently by Wong *et al.* (2), who showed that interaction *in vitro* between tumor cells and spleen cells caused the spleen cells to become immunologically unresponsive, and by Fauve *et al.* (3), who reported that tumors repulse macrophages *in vitro* and inhibit the inflammatory reaction *in vivo*.

It seems, therefore, that subversion of the immune system by tumors could be a viable escape mechanism for tumors. However, unless we know the mechanism of this subversion and how to control it, we cannot hope to use immunotherapy effectively in treating cancer. Starting with the clue that tumor cells tend to produce excessive amounts of prostaglandins (4–6), we have obtained evidence that subversion of the immune system may be mediated by prostaglandins and that inhibitors of prostaglandin synthetases, such as indomethacin and aspirin, could be useful in counteracting subversion of the immune system by tumors.

Abbreviations: $PGE_2$, prostaglandin $E_2$; MCDV-12, a mouse ascites tumor line induced in the BALB/c strain by Rauscher leukemia virus; MC-16, mouse fibrosarcoma induced in the C57B1/6J strain by the carcinogen, methylcholanthrene; sRBC, sheep erythrocytes.

## MATERIALS AND METHODS

*Tumor Cell Lines.* An ascites cell line (MCDV-12), induced in BALB/c mice by Rauscher leukemia virus, was obtained from the National Cancer Institute and maintained by serial passage in BALB/c mice at 5-day intervals. A solid fibrosarcoma (MC-16), induced by us in C57B1/6J mice by methylcholanthrene, was passaged serially in C57B1/6J mice at 21-day intervals. A suspension of MC-16 tumor cells was prepared by mincing freshly excised tumor, after removal of connective and necrotic tissue, with a scalpel, digesting with collagenase, and recovering the dissociated cells by filtration through a gauze. Cells were counted and tested for viability by exclusion of Trypan Blue.

*Mice.* The C57B1/6J mice, used for passaging the MC-16 tumor and for testing immunological responsiveness while bearing a tumor, were 6-weeks-old female mice purchased from Jackson Laboratories, Bar Harbor, Me. The BALB/c mice, also used for passaging syngeneic tumor and for testing immunological responsiveness, were 6-weeks-old females purchased from Cumberland Farms, Clinton, Tenn.

*Test of Mice for Immunological Competence.* Competence was measured in terms of antibody response to sheep erythrocytes (sRBC). Stock sheep blood, purchased from Colorado Serum Co., Denver, Colo, was used as a source of sRBC.

Test mice, normal controls, as well as tumor-bearers, were given $10^8$ sRBC intraperitoneally and at defined times thereafter, their antibody response was assessed. Sera were assayed for hemagglutinating antibody by means of a microtiter test system, and the spleens were assayed for antibody-producing cells by means of the hemolytic plaque-forming cell assay of Jerne (7).

The alternative to injecting sRBC into mice was to add sRBC to *in vitro* cultures of spleen cells from test mice and to measure the antibody response in terms of the development of plaque-forming cells (PFC). This is essentially the Mishell–Dutton system of inducing antibody formation to sRBC *in vitro*, and their procedure (8), modified according to Click *et al.* (9), was used.

## RESULTS

### Immunosuppression associated with tumorigenesis *in vivo*

Groups of BALB/c mice were administered $10^3$ syngeneic MCDV-12 cells intraperitoneally; this is sufficient to cause death in about 2 weeks. Control mice received diluent, pyrogen-free saline. At intervals of 2 days thereafter, groups of

TABLE 1. *Immunosuppression associated with tumorigenesis in BALB/c mice*

| Group | Syngeneic virus-induced tumor implanted on day 0 | sRBC injected on day | Antibody response* Plaque-forming cells/$10^6$ spleen cells | Antibody response* Hemagglutinin titer |
|---|---|---|---|---|
| 1A | − | 0 | 2580 | 7.0 |
| 1B | + | 0 | 1960 | 7.5 |
| 2A | − | 2 | 3040 | 7.0 |
| 2B | + | 2 | 3240 | 7.0 |
| 3A | − | 4 | 1960 | 7.0 |
| 3B | + | 4 | 348 | 5.5 |
| 4A | − | 6 | 1120 | 7.0 |
| 4B | + | 6 | 450 | 1.0 |

* Four days after administration of sRBC, mice were first bled to obtain serum and then sacrificed to obtain the spleen. Sera were assayed for hemagglutinating antibody; the titer is expressed as $\log_2$ of dilution end-point. Spleens were assayed for antibody-forming cells, measured as plaque-forming cells.

these mice were tested for immunological responsiveness to sRBC, injected intravenously. In all cases, they were examined for antibody response 4 days after injection of sRBC. The sera from these mice were assayed for hemagglutinating antibody, and their spleens were assayed for plaque-forming cells. The results, given in Table 1, show that by day 4, tumor-bearing mice were already significantly immunodeficient and, by day 6, essentially unresponsive to sRBC.

In a similar experiment, $10^6$ syngeneic MC-16 tumor cells were inoculated into groups of C57Bl/6J mice. This number of cells sufficed to establish a tumor that was generally palpable in about 1 week and that grew progressively until death of the animals in about 4 weeks. At intervals of 5 days, starting from the time tumor cells were inoculated, groups of these mice were tested for immunological responsiveness to sRBC. Instead of injecting the sRBC directly into the animals and measuring the antibody response *in vivo*, as in the previous experiment, the sRBC were added to suspensions of spleen cells from the test animals and cultured *in vitro* for 4 days, after which the cultures were examined for plaque-forming

TABLE 2. *Changes in responsiveness to sRBC of spleen cells from C57Bl/6J mice after inoculation of syngeneic chemically-induced tumor*

| Day after tumor inoculation* | Antibody response (% of control) |
|---|---|
| 5 | 93 |
| 10 | 16 |
| 15 | 1 |
| 20 | 1 |

* At times indicated, groups of mice were sacrificed and their spleens used to prepare suspensions of cells which were cultured *in vitro* with sRBC for 4 days; the immune responsiveness of the spleen cells was then assessed by comparing the number of plaque-forming cells produced by these animals with the number produced in cultures of spleen cells from normal non-tumor-bearing mice.

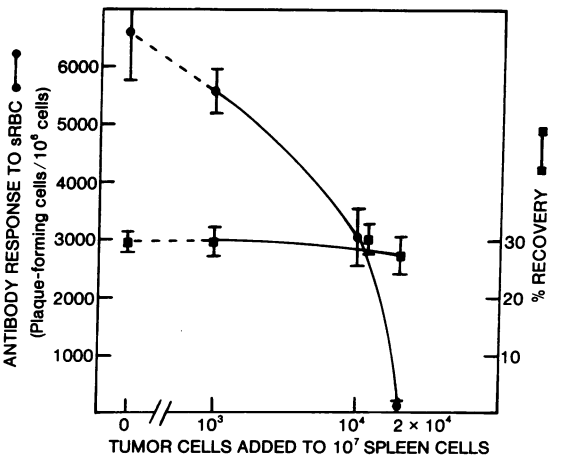


FIG. 1. Immunosuppressive property of syngeneic methylcholanthrene-induced tumor cells (MC 16). Tumor cells were added to *in vitro* cultures of syngeneic C57Bl/6J spleen cells and sRBC. After 4 days the cultures were examined for plaque-forming cells and viable spleen cells.

cells. In each instance, spleens of normal mice served as controls. The tumor-bearers were almost completely immunosuppressed by day 10 as is clearly shown in Table 2 and judging from its rate of development, immunodeficiency had probably started on about day 5.

### Immunosuppression by tumor cells *in vitro*

The above results suggested a possible causal relationship between immunosuppression and tumorigenesis. To test this possibility, we added MC-16 tumor cells directly to syngeneic spleen cells, cultured these *in vitro with* sRBC for 4 days, and then examined the cultures for antibody producing plaque-forming cells and viable nucleated spleen cells. A number of such cultures were set up with various numbers of tumor cells. It is evident (Fig. 1) that tumor cells were not toxic to the spleen cells but did, nevertheless, suppress their antibody response to sRBC as a consequence of direct interaction. The fact that as few as 1 tumor cell per 1000 spleen cells sufficed to produce significant suppression of antibody response reveals the highly subversive character of these tumor cells.

### Immunosuppression by prostaglandins

Prostaglandins, particularly prostaglandin $E_2$ ($PGE_2$), are reportedly produced in excess amounts by certain tumor cell lines (4–6). The possibility was considered, therefore, that prostaglandins might be the responsible immunosuppressive agent of the tumor cells tested above. Accordingly, $PGE_2$ (obtained from Dr. Pike, Upjohn) was tested for immunosuppressive activity directly by adding it to *in vitro* cultures of spleen cells and sRBC. As before, the cultures were maintained for 4 days and then examined for plaque-forming cells. As shown in Table 3, $PGE_2$ was indeed immunosuppressive.

Another test of the possible role of prostaglandins in immunosuppression by tumor cells was to add indomethacin, an inhibitor of prostaglandin synthetases, to *in vitro* cultures consisting of immunosuppressive tumor cells, spleen cells, and sRBC, and to look for inhibition of suppression of the antibody response of spleen cells to sRBC. The indomethacin (obtained from Dr. Kuehl, Merck and Co.) was added to the cul-

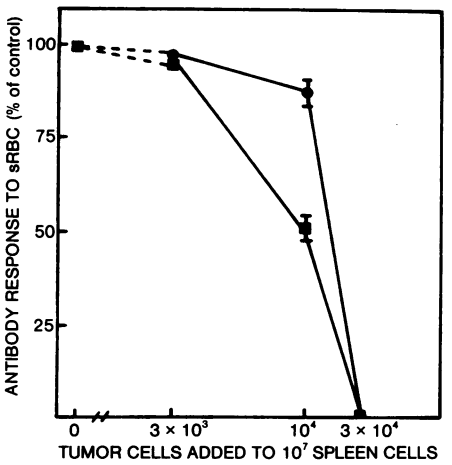


FIG. 2. Test of indomethacin for ability to block immunosuppression by tumor cells. MC 16 tumor cells were added to *in vitro* cultures of syngeneic C57B1/6J spleen cells and sRBC. To one set of cultures indomethacin (5 μg/ml) was added (●), and to a second set of cultures diluent was added (■). After 4 days, all cultures were examined for plaque-forming cells. % of control = number of plaque-forming cells in experimental cultures/number of plaque-forming cells in control cultures ×100. The standard error for $10^4$ tumor cells is shown, with $P < 0.001$.

tures at a final concentration of 5 μg/ml. At this concentration it was not toxic to either the tumor cells or the spleen cells (10). However, low concentrations of tumor cells were less immunosuppressive in the presence of indomethacin (Fig. 2). Aspirin, which also inhibits prostaglandin synthetases, gave

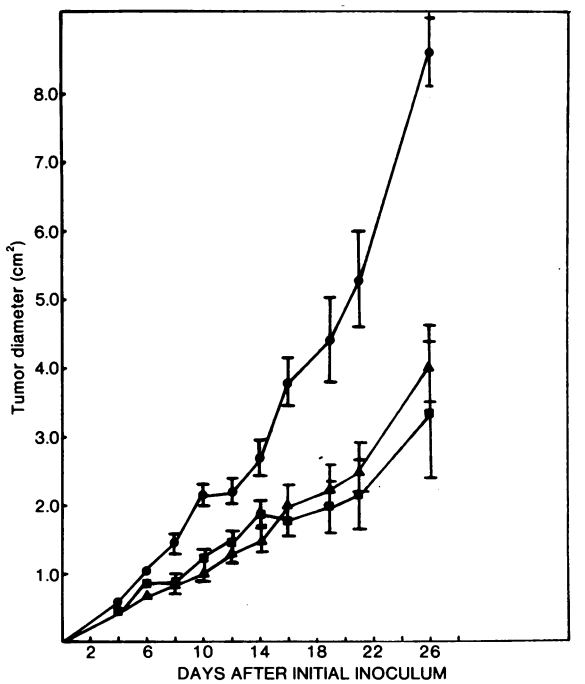


FIG. 3. Effect of indomethacin on growth of MC 16 tumor cells in syngeneic C57B1/6J mice. Tumor cells ($10^6$) were injected subcutaneously into three groups of mice (10/group). One group received indomethacin intraperitoneally (125 μg/day throughout first 10 days) (■); a second group also received indomethacin but for a 14-day period (▲); the third group served as control and received diluent (●). Tumor mass is expressed as mean diameter ($cm^2$) ±SE.

TABLE 3. *Immunosuppression by prostaglandin $E_2$ ($PGE_2$) of spleen cells from C57B1/6J mice*

| μg $PGE_2$/ml of culture* | Antibody response to sRBC (% of control) |
|---|---|
| 0 | 100 |
| 0.0001 | 105 |
| 0.001 | 80 |
| 0.01 | 85 |
| 0.1 | 68 |
| 1.0 | 60 |
| 10.0 | 49 |

* $PGE_2$, in amounts indicated, was added to *in vitro* cultures of spleen cells; sheep erythrocytes were added 15 min later. After 4 days, the antibody response was measured by examining the cultures for plaque-forming cells.

similar results (data not shown), and provides further evidence for involvement of prostaglandins in immunosuppression by tumor cells.

Because indomethacin blocked immunosuppression by tumor cells *in vitro*, it was also tested for its possible effect on tumor development *in vivo*. Groups of C57B1/6J mice were inoculated with syngeneic MC-16 tumor, and treatment with indomethacin was started on the day of tumor inoculation. The control group was given diluent, and two experimental groups were given indomethacin, one for 10 days and the other for 14 days. The rate of tumor growth was significantly reduced as a result of indomethacin treatment, but it was not arrested even when the period of treatment was extended beyond 10 days into the period of increased rate of tumor growth (Fig. 3).

## DISCUSSION

The immunological competence of mice, as measured by their antibody response to sRBC, decreased dramatically during the course of tumor growth. Since this was true for both chemical and virus-induced tumor cell lines, immunosuppression may be a property of malignant cells generally, without regard to etiology. However, a survey of a larger number of transplantable tumors is clearly needed. That tumor cells are the causative agents of immunosuppression is based on the finding that direct interaction, in *in vitro* culture, between tumor cells and immunologically competent spleen lymphocytes resulted in suppression of the antibody response of the spleen cells to sRBC. This suppression was dose dependent, and tumor cells added to spleen cells in a ratio of 1 to 1000 caused significant immunosuppression, thus revealing the highly subversive nature of tumor cells. Wong *et al.* (2) have recently reported a similar finding.

We also have evidence that tumor cells need to be viable and metabolically active in order to be immunosuppressive (10), and there have been reports that certain tumor cell lines produce excessive amounts of the prostaglandin $PGE_2$ (4–6) and the $PGE_2$ stimulates endogenous adenosine 3′:5′-cyclic monophosphate (cAMP) in spleen cells (11). For the above reasons, the possible role of prostaglandins in immunosuppression by tumor cells was investigated. Indeed, $PGE_2$ proved to be immunosuppressive, and indomethacin and aspirin, which share the property of inhibiting prostaglandin synthetases, blocked significantly the immunosuppressive activity of tumor cells. The fact that indomethacin was also

able to retard tumor growth *in vivo* is further evidence of the possible role of prostaglandins in tumor immunosuppression. Additional evidence has been obtained by Strausser and Humes (12), who found that indomethacin, administered to immunologically competent mice at the time they were inoculated with Moloney sarcoma virus, prevented the development of tumors. The fact that indomethacin was effective only in immunologically competent mice argues against indomethacin being a toxic antitumor agent. These results further support our conclusion that indomethacin may act to block immunosuppression mediated by prostaglandins; the immune system of the host thus prevents the development of strongly antigenic tumors and retards the development of weakly antigenic tumors.

Involvement of $PGE_2$ in tumor immunosuppression is based on the evidence that $PGE_2$ is itself immunosuppressive and that inhibitors of prostaglandin synthetases block tumor immunosuppression *in vitro* and affect tumor development *in vivo*. Nevertheless, $PGE_2$ probably is not the only mediator of immunosuppression by tumor cells because $PGE_2$ did not suppress the antibody response completely even at high concentrations, and inhibitors of prostaglandin synthetases did not block immunosuppression when a relatively large number of tumor cells was tested. The actual role of prostaglandins in tumor immunosuppression can best be assessed when we have completed examining a number of transplantable tumor lines for possible correlation between their capacity to synthesize $PGE_2$ and their ability to subvert the immune system. cAMP might well be the actual transmitter of tumor immunosuppression because both tumor cells and $PGE_2$ cause elevation of the cAMP level of spleen cells on interaction (11, 13), and we know that changes in cAMP are associated with activation and function of immune cells (14).

We have presented here direct evidence that syngeneic tumors can subvert the humoral response to an antigen, but the relevance of this finding to tumorigenesis could be questioned since tumor immunity is primarily cellular rather than humoral. However, we have reason to believe that the cellular immune response is also subject to subversion by syngeneic tumors. Preliminary results indicate that mice bearing a syngeneic tumor become increasingly unresponsive to a viable tumor allograft; the tumor implant is rejected more slowly or not at all and, in those instances in which regression of the tumor occurs, a new tumor arises at the site of implantation apparently as a result of continuing subversion of the immune system by the growing syngeneic tumor. Thus, cells participating in both humoral and cellular immune responses are apparently subverted by a syngeneic tumor.

Recently, Fauve *et al.* (3) reported that murine malignant cells can subvert macrophages by repulsing them and thereby prevent them from attacking the tumor. They also reported that tumors produce and release a substance that inhibits local inflammatory reactions around the tumor. This represents yet another type of host defense cell that is subject to subversion by a syngeneic tumor, but the mediator of this subversion of macrophages is probably not prostaglandin because the reported molecular weight of the antiinflammatory factor is in excess of 1000.

The fact that immunodeficiency is associated with tumorigenesis is an important phenomenon, whether or not we know or understand fully the underlying cause or basis for it, because antigenic tumors can surely develop more readily in immunodeficient hosts than in competent ones. Thus, tumor cells, having developed a capacity for immunosuppression, may have evolved an efficient means of escaping immunosurveillance. If it is true, as our data indicate, that tumors are able to establish themselves despite their antigenicity, because they can subvert the immune system, it is clear that immunotherapy, based on specific vaccines or on nonspecific stimulation of the immune system, would be handicapped unless continuing immunosuppression by the tumor itself is prevented or blocked. Removal of tumor by surgery is effective in preventing further immunosuppression and it permits restoration of immunological competence (10). The problem is what measures to take if surgery is not feasible. The only answer would seem to be to use chemotherapy, but the objectives of chemotherapy should be multi-faceted: to prevent or block immunosuppression by tumor itself, to stimulate the immune system, and to contain the tumor mass to a level that is manageable by the immune system. We already have numerous anti-tumor and immunostimulating drugs. What seems to be needed are drugs that can block the subversive activity of tumor cells. If $PGE_2$ proves to be a significant factor in subversion, indomethacin could be effective because it is a potent inhibitor of prostaglandin synthetases, and unlike certain other types of anti-inflammatory drugs, indomethacin is not immunosuppressive (15).

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